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ORIGINAL PAPER

Evidence for genetic differentiation and divergent selection in an autotetraploid forage grass (*Arrhenatherum elatius*)

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Abstract The use of local provenances in restoration, agriculture and forestry has been identified as measure to sustain biological diversity and to improve local productivity. However, the delineation of regional provenances is challenging because it requires the identification of well-defined groups based on spatiogenetic differentiation and/or the evidence of local adaptation. In this study, we investigate genetic variation at 186 AFLP loci in 46 European accessions of the important grassland species *Arrhenatherum elatius* and ask (1) whether genetic variation within accessions differs between European geographical regions; (2) at which spatial scale populations are structured across Europe and (3) whether putatively adaptive markers contribute to this pattern and whether these markers can be related to climatic site conditions. Basic expectations of population genetics are likely to be altered in autotetraploid species, thus, we adopted a band-based approach to estimate genetic diversity and structuring. Compared to other grasses *A. elatius* showed high genetic diversity and considerable differentiation among accessions ($\Phi_{ST} = 0.24$). Accessions separated in a Western European and a Central/Eastern European group, without further structure within groups. A genome scan approach identified four potentially adaptive loci, whose band frequencies correlated significantly with climatic parameters, suggesting that genetic differentiation in *A. elatius* is also the result of adaptive processes. Knowledge on adaptive loci might in the long run also help to adapt ecosystems to adverse climate change effects through assisted migration of ecotypes rather than introduction of new species.

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Introduction

A large amount of seed and plant material is released into the open landscape by means of compensatory measures, the cultivation of forage or energy plants, the sowing of grassland areas or roadside greenery and plantings in the course of restoration. This practice is not new as the intentional transfer of significant quantities of seeds can be traced back to at least two or three hundred years ago when grasslands were enhanced by seeds of specific forage grasses (Kauter 2002). When seed and plant material is used without regard for the provenance, these activities may introduce new species to local communities or may introduce genes and genotypes not present in local populations. The introduction of different genotypes has been

recognized as a potential risk for regional genetic and species diversity for several reasons (Hufford and Mazer 2003; Bleeker et al. 2007). Foreign genotypes may not be adapted to site conditions, genetic swamping may lead to extinction of remnant local genotypes, and outbreeding depression may occur in hybrids between native and introduced individuals (Anttila et al. 2000; Hufford and Mazer 2003; Prentis et al. 2007). Consequently, the use of regional provenances in planting, sowing and stocking activities has been increasingly demanded and supported by law and policy (Sackville Hamilton 2001; Mijnsbrugge et al. 2009). The consistent use of regional provenances should assist a greater resistance of the introduced plant material, resulting from adaptation to local conditions, which has been demonstrated for many species, traits and different scales (Joshi et al. 2001; Bischoff et al. 2006; Hufford et al. 2007). The consistency in local population dynamics and the minimization of human interference by using regional provenances should ensure the functioning of species communities, the maintenance of ecosystem services and conserves naturally evolved biodiversity (cf. Hooper et al. 2005). However, one challenge in using regional provenances is the definition of what is regional (McKay et al. 2005). Whereas for forest trees, species specific seed transfer zones have been identified based on extensive analyses of stress resistance and adaptation (e.g., Saenz-Romero and Tapia-Olivares 2008), similar effort is hardly undertaken for grassland species. A general approach to delineate regional provenances is to identify areas with similar climatic and ecological conditions. Albeit this may serve as a first step for practical purposes if other information is not available it does not consider species-specific patterns of genetic differentiation and adaptation that may depend on gene dispersal characteristics, migration history and the species-specific response to selective forces. Thus, the delineation of regional provenances requires the identification of groups of individuals based on spatiogenetic differentiation and/or the evidence of local adaptation (Kleinschmit et al. 2004). Regional provenances might also help to identify ecotypes which are already adapted to climatic conditions expected in the future for locations further north within the species' current distribution. This approach would secure ecosystem integrity by conserving the presence of key species such as perennial grasses or forest trees.

Arrhenatherum elatius (L.) P. Beauv. ex J. Presl & K. Presl (Poaceae) is a significant forage grass and as such introduced worldwide. *A. elatius* is native in, and widely distributed throughout Europe. It can be found in eutrophic grasslands from low altitudes up to over 2,000 m in the Alps or even higher in the Caucasus (Pfitzenmeyer 1962). It has been hypothesized that the species is not native in Central Europe but introduced following an increase in cultivation of grasslands at the end of the middle-ages or even later (Buch et al. 2007 and references therein). However, fossil evidence backs an earlier distribution of the species in Central Europe (reviewed in Kauter 2002). A first cultivation of *A. elatius* may have occurred in Southern France as early as in the sixteenth century, from where seed material was later widely distributed in Europe as 'French Rye' (Conert 1998; Kauter 2002). The species is frequently included in seed mixtures for pastures and used in restoration measures.

The high phenotypic variability that has been described for *A. elatius* (Jenkin 1931; Sulinowski 1965a; Mahmoud et al. 1975) and the degree of genetic variation that has been found at smaller scales for few populations of the species (Ducousso et al. 1990; Petit et al. 1997; Petit and Thompson 1998) may suggest a strong potential for adaptive evolution. Directional selection may very quickly lead to adaptive genetic changes (Jump et al. 2008), thus, also in regions with a quite recent species history, as possibly *A. elatius* in Central Europe, genetic differentiation can be the result of adaptive processes.

In this study, we investigate genetic variation at AFLP loci in *Arrhenatherum elatius* and ask whether patterns of neutral or putatively adaptive genetic variation suggest the delineation of regional provenances. Specifically we ask (1) whether genetic variation within accessions differs between European regions; (2) at which spatial scale populations are genetically structured across Europe and (3) whether putatively adaptive markers contribute to this pattern and whether these markers can be related to environmental site conditions.

Materials and methods

Study species

Arrhenatherum elatius is a perennial, tussock-forming grass covering a wide range of ecological conditions. The species is of autotetraploid origin ($2n = 4x = 28$) with few diploid exceptions (Petit et al. 1997; Conert 1998). The species is wind-pollinated and has been described as mainly outcrossed, but varying degrees of self-fertility were reported from bagging experiments (Sulinowski 1965b, and references therein; Cuguen et al. 1989). Outcrossing rates based on progeny analysis of two tetraploid populations indicate a mixed mating system ($t_m = 0.541$ and 0.701, Petit et al. 1997). Sexual reproduction has been described as severely handicapped by cold climates (Pfitzenmeyer 1962). Vegetative reproduction by 'bulbs', swollen basal stem internodes, may occur in individuals of *A. elatius* var. *bulbosum* (Willd.) Spenn. under suitable conditions, e.g., in arable fields. Otherwise, reproduction is by seed.

Sampling, DNA extraction and AFLP analysis

Leaf tissue was sampled from plants (*A. elatius* var. *elatius*) grown from seed material provided by gene banks of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben/Germany, the Institute for Agrobotany in Tápiószele/Hungary, the Crop Research Institute (RICP) in Prague-Ruzyne/Czech Republic. One accession was directly sampled near Bayreuth, Germany. The accessions were initially sampled from 46 populations of *A. elatius* across Europe (Table 1) and were grouped into four geographical groups: South Western Europe (5 Spanish accessions), North Western Europe (5 Irish), Central Europe (18 accessions from France, Denmark, Germany) and Eastern Europe (18 accessions from Poland, Czech Republic, Hungary, Russia; Fig. 1). Per accession, leaf tissue from up to 11 individuals was freeze-dried and genomic DNA was extracted with the DNeasy 96 Plant extraction kit (QIAGEN, Hilden, Germany).

We analyzed amplified fragment length polymorphisms (AFLP, Vos et al. 1995). For restriction and ligation 6 µl of template DNA (approx. 500 ng DNA) were combined with 5 µl of restriction-ligation (RL) reaction mix containing 0.05 µl H_2O (HPLC-grade, Merck), 0.55 µg BSA (New England Biolabs, NEB), 0.11 M NaCl, 5 u *Eco*RI (NEB), 1 u *Mse*I (NEB), 1.1 µl T4 DNA ligase buffer (NEB), 67 u T4 DNA ligase (NEB), 1 µl *Mse*I adapter (50 mM) and 1 µl *Eco*RI adapter (5 mM). The reaction was incubated 2 h at 37°C and diluted 1:5. For preselective amplification 4 µl of RL template were combined with 16 µl of preselective PCR reaction mix containing 1.5 ng/µl of *Mse*I- and *Eco*RI preselective primers each, 200 µM dNTPs (Roth), 2 µl PCR buffer + $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.8 u Taq polymerase (Fermentas) and 9.64 µl H_2O. The thermocycler protocol was 72.0°C (2 min) followed by 20 cycles of 94.0°C (20 s), 56.0°C (30 s) and 72.0°C (2 min) and a final step at 60.0°C (30 min). For selective amplification, 1 µl of the preselective PCR products (diluted 1:5) was added to 2.2 µl of Multiplex PCR kit (QIAGEN) and 0.6 µl of *Mse*I (5 mM) and 0.6 µl *Eco*RI (1 mM) selective primers. The thermocycler protocol was 94.0°C (2 min) followed by 10 cycles of 94.0°C (20 s), 66.0°C (30 s, decreasing 1°C per cycle) and 72.0°C (2 min) and 20 cycles of 94.0°C (20 s), 56.0°C (30 s) and 72.0°C (2 min), and a final step at 60.0°C (30 min). After screening of eight primer combinations on three individuals from four accessions each, four primer combinations were selected for the analyses (Table 2). AFLP fragments were separated on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, USA) with internal size standard Genescan 500 LIZ (Applied Biosystems). Individuals were genotyped using GeneMapper version 3.7 (Applied Biosystems). Only unambiguously scoreable fragments were translated into a binary matrix for further analysis.

Genetic variation and population genetic structure

In contrast to diploid or allopolyploid species, in autotetraploid species the segregation pattern of chromosomes during meiosis is tetrasomic and thus much more complex. Here, the two sister chromatids may segregate into the same gamete leading to an increased production of homozygous gametes as compared to what is expected under random segregation ("double reduction", Bever and Felber 1992; Ronfort et al. 1998). Moreover, the degree of double reduction may vary among loci. Thus, basic expectations of population genetics are very likely to be altered in species exhibiting tetrasomic inheritance (Bever and Felber 1992). Due to this fact and because AFLPs are dominant markers we strictly adopted a simple band-based approach to estimate genetic diversity and structuring rather than methods based on the estimation of allele frequencies. However, homoplasy in AFLP data might be, to a certain degree, unavoidable (Gaudeul et al. 2004) and may introduce bias. When using a phenetic approach to analyze AFLP data, common band absence is possibly the most likely source of homoplasy, at least when the degree of genetic similarity among individuals is low (Kosman and Leonard 2005). To overcome potential bias by homoplasy, we estimated genetic diversity and differentiation based on the Jaccard similarity coefficient (S). This index considers only band presence as informative and has been applied successfully in similar studies (cf. Subudhi et al. 2005; Cavagnaro et al. 2006). A pairwise distance matrix between all sampled individuals was computed as $1 - S$ using the package VEGAN (Oksanen et al. 2008) for the R 2.7.2 environment (R Development Core Team 2008), in which also most of the subsequent statistical analyses were conducted. Genetic diversity was evaluated for each accession as the mean pairwise distance between its individuals (J). Differences in genetic diversity between geographical groups of accessions were analyzed by ANOVA and Tukey's post hoc tests. A differentiation measure (D_J) between accessions was obtained by averaging distances of all pairwise comparisons between individuals of two accessions at a time. Significance was defined by deviation from a null distribution, obtained by random permutation of the individuals of both respective accessions 1,000 times, when the observed distance was greater than 95% of the values drawn from the null distribution. A dendrogram of accessions was constructed using pairwise D_J values as input for the Neighbor-Joining algorithm implemented in the program 'Neighbor' of the PHYLIP Package version 3.6 (Felsenstein 2004). Reliability of clusters was tested by bootstrapping over loci 1,000 times.

Population genetic structure was evaluated by analysis of molecular variance (AMOVA) based on the Jaccard distance matrix between individuals as described above using

Table 1 Origin and genetic diversity parameters derived from 186 AFLP loci of 46 accessions of *Arrhenatherum elatius*

Accession	Accession-ID	Latitude (°N)	Longitude	Sample size	# of polymorphic loci	Proportion of polymorphic loci	*J*
Western Europe–Spain							
ESP01	GR 7127/2001	43.26	−7.29	10	136	73.1	0.389
ESP02	GR 7129/2001	43.05	−8.28	8	136	73.1	0.399
ESP03	GR 7130/2001	42.30	−7.87	10	131	70.4	0.408
ESP04	GR 7131/2001	42.63	−8.12	11	148	79.6	0.372
ESP05	GR 7133/2001	43.23	−8.02	10	133	71.5	0.343
Mean				9.8	137	73.5	0.382
Western Europe–Ireland							
IRL01	GR 11811/2004	53.36	−6.33	10	128	68.8	0.264
IRL02	GR 12056/2005	52.64	−8.95	11	141	75.8	0.396
IRL03	GR 12058/2005	53.52	−8.85	11	156	83.9	0.405
IRL04	GR 12059/2005	51.55	−9.27	11	147	79.0	0.390
IRL05	GR 12066/2005	52.06	−9.51	10	138	74.2	0.353
Mean				10.6	142	76.3	0.362
Central Europe							
DNK01	GR 7293/2002	55.79	11.51	10	143	76.9	0.351
FRA01	GR 11704/2003	44.81	5.93	9	148	79.6	0.370
GER01	GR 313/2002	50.55	10.79	10	125	67.2	0.297
GER02	GR 314/1998	50.61	10.69	9	137	73.7	0.341
GER03	GR 316/2002	50.57	10.81	10	125	67.2	0.288
GER04	GR 317/1998	50.55	10.79	10	158	84.9	0.431
GER05	GR 331/2003	51.85	13.75	10	140	75.3	0.351
GER06	GR 341/2003	51.75	10.76	10	130	69.9	0.317
GER07	GR 342/1998	51.64	10.98	10	136	73.1	0.330
GER08	GR 344/1998	51.89	12.03	10	134	72.0	0.317
GER09	GR 350/1998	51.93	14.09	7	134	72.0	0.384
GER10	GR 357/2003	51.08	11.00	9	137	73.7	0.392
GER11	GR 367/1997	54.32	13.53	6	130	69.9	0.335
GER12	GR 7259/2002	51.45	11.92	9	155	83.3	0.404
GER13	GR 7260/2002	51.34	12.37	10	142	76.3	0.335
GER14	GR 7261/2002	52.08	12.23	10	144	77.4	0.339
GER15	GR 7271/2002	52.51	13.12	10	152	81.7	0.382
GER16	*7/2006	49.30	9.97	8	155	83.3	0.437
Mean				9.3	140	75.4	0.356
Eastern Europe							
CZE01	14G0700045	48.93	17.85	10	157	84.4	0.381
CZE02	14G0700046	48.94	17.78	10	147	79.0	0.340
CZE03	14G0700044	48.85	17.40	8	151	81.2	0.356
CZE04	14G0700043	48.91	17.55	9	154	82.8	0.375
CZE05	14G0700064	48.81	16.05	6	148	79.6	0.390
HUN01	RCAT042238	46.58	20.49	10	146	78.5	0.389
HUN02	RCAT064779	47.25	20.54	10	155	83.3	0.410
HUN03	RCAT040667	47.43	18.23	10	152	81.7	0.359
HUN04	RCAT040668	47.68	16.57	10	157	84.4	0.423
HUN05	RCAT041661	47.51	18.14	9	152	81.7	0.353
HUN06	RCAT041156	47.63	18.46	10	156	83.9	0.386
POL01	GR 339/1998	50.57	21.68	10	151	81.2	0.353

Table 1 continued

Accession	Accession-ID	Latitude (°N)	Longitude	Sample size	# of polymorphic loci	Proportion of polymorphic loci	*J*
POL02	GR 363/2003	49.83	20.31	9	143	76.9	0.391
POL03	GR 364/2003	49.99	22.46	10	131	70.4	0.402
POL04	GR 366/2002	49.82	20.30	10	120	64.5	0.311
POL05	GR 5516/2003	51.14	16.87	8	137	73.7	0.376
POL06	GR 5976/2004	53.03	18.46	9	140	75.3	0.393
RUS01	14G0700091	54.73	21.40	10	148	79.6	0.381
Mean				9.3	147	79.0	0.376
Mean all				9.5	143	76.9	0.365

Accession-ID indicates from which gene bank samples were taken: GR, IPK, Gatersleben/Germany; RCAT, Institute for Agrobotany, Tápiószele/Hungary; 14G, Crop Research Institute, Drnovská/Czech Republic. The asterisk indicates an accession stored at the Faculty of Biology, University Bayreuth/Germany. The provenance code indicates the country of origin: ESP, Spain; IRL, Ireland; GER, Germany; CZE, Czech Republic; FRA, France; POL, Poland; HUN, Hungary. Genetic diversity of accessions is measured by the number and proportion of polymorphic loci, and by the mean pairwise Jaccard dissimilarity among individuals within accessions (*J*)

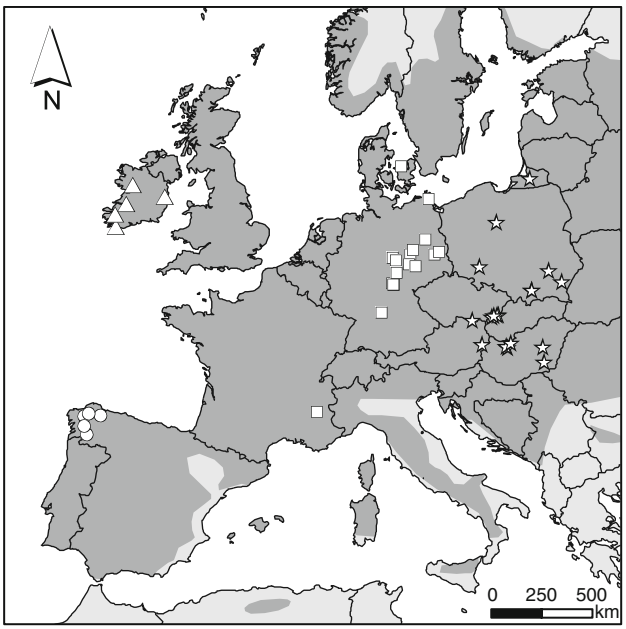

Fig. 1 Geographical origin of the 46 accessions of *Arrhenatherum elatius* investigated. *Symbols* represent different regional groups (*circles* Spanish accessions, *triangles* Irish accessions, *squares* Central European accessions, *stars* Eastern European accessions). The area marked in darker *gray* represents the natural distribution of *A. elatius* in Europe

Table 2 Primer sequences used for preselective and selective amplification

Primer	Sequence
Adapters	
*Eco*RI-adapter	
Top	5′ CTCGTAGACTGCGTACC 3′
Bottom	3′ CATCTGACGCATGGTTAA 5′
*Mse*I-adapter	
Top	5′ GACGATGAGTCCTGAG 3′
Bottom	3′ TACTCAGGACTCAT 5′
Preamplification primers	
*Eco*RI-E01	5′ GACTGCGTACCAATTCA 3′
*Mse*I-M01	5′ GATGAGTCCTGAGTAAC 3′
Selective amplification primers	
*Eco*RI-ACT FAM[1]	5′ FAM-GACTGCGTACCAATTCACT 3′
*Eco*RI-ACA VIC[2]	5′ VIC-GACTGCGTACCAATTCACA 3′
*Eco*RI-ACC NED[3]	5′ NED-GACTGCGTACCAATTCACC 3′
*Eco*RI-AGC PET[4]	5′ PET-GACTGCGTACCAATTCAGC 3′
*Mse*I-CTT[1,4]	5′ GATGAGTCCTGAGTAACTT 3′
*Mse*I-CTA[2,3]	5′ GATGAGTCCTGAGTAACTA 3′

Superscript numbers indicate primers used together in combination for selective amplification

Arlequin 3.0 (Excoffier et al. 2005). Covariance components were computed for three comparisons: (1) Including all four spatial groups of accessions, (2) including only the two groups from Western Europe and (3) including only Central and Eastern Europe.

To test for equilibrium between gene flow and drift pairwise genetic and spatial distances between accessions were correlated on the global scale including all accessions and on a more regional scale by using either all Central and Eastern European accessions or only the Western European accessions. Significance of all correlations was evaluated by Mantel tests running 10,000 permutations.

Detection of loci potentially under selection

To evaluate a possible adaptive genetic divergence among accessions, environmental parameters were related to band frequency at single marker loci by logistic regression analysis. Annual growing degree days [GDD (°C)], mean annual temperature [*T* (°C)], annual precipitation [*P* (mm)] and annual potential evapotranspiration ([PET (mm)] as well as

the ranges of mean monthly temperatures (range T) and precipitation (range P) were averaged across the time period of 1971–2000. Data were extracted for each accession from Mitchell et al. (2004). Environmental parameters were partly intercorrelated (e.g., GDD and T: $r = 0.91$, $P < 0.001$; P and range T: $r = -0.79$, $P < 0.001$). To reduce dimensions and eliminate collinearity, we applied a principal component analysis (PCA) using the package ADE4 (Dray and Dufour 2007) for R. The two factors that accounted most for the variation in the environmental parameters (87%) were used subsequently in the regression analysis.

Relating all AFLP loci individually to the two extracted factors might result in increased type I error and inflated number of significant outcomes. Hence, a preselection on all loci was accomplished by running the DFDIST program (http://www.rubic.rdg.ac.uk/~mab/stuff/), a modification for dominant markers of software developed by Beaumont and Nichols (1996). This software identifies loci that potentially are under selection by comparing empirical F_{ST} values for each locus against a null distribution of F_{ST} values expected from a neutral drift model. As this software is designed for diploids, it is necessary to consider the differences between different ploidy levels, all else being equal. First, in theory, the effect of drift on population structure in tetraploids is reduced compared to diploids due to a doubled effective population size. Second, in species with tetrasomic inheritance genetic differentiation between populations can either be increased or decreased compared to species with disomic inheritance depending on the occurrence of selfing and double reduction during meiosis (Ronfort et al. 1998). For species with tetrasomic inheritance, if selfing is low and double reduction does not occur at a certain locus, this locus may go undetected by the screening procedure, although under selection. Although differentiation at that locus would be higher than that expected as neutral, it might be still lower than the degree of neutral divergence that can be accumulated by diploids and that is estimated by DFDIST, because of the effects of the doubled effective population size. On the other hand, loci prone to double reduction but being not adaptive, may lay outside a frequently applied 95% confidence interval for neutral divergence in diploids because double reduction increases genetic divergence. We reduced the number of these 'false positives' by applying a very conservative significance level of 99.99%, while potentially underestimating the fraction of adaptive loci that not undergo double reduction.

For each preselected locus, AFLP band frequencies per accession were explained by the two environmental factors (PCs) via separate logistic regression models with binomial error distributions. To account for overdispersion, a quasi-likelihood estimation approach was applied. The regression procedure was weighted by the number of samples in each accession. Only those relationships were considered significant that remained so after Bonferroni correction for multiple tests. To detect potentially adaptive loci on a more regional scale a second analysis was conducted including only accessions from Central and Eastern Europe.

Results

Genetic diversity within and among accessions

Some samples failed to amplify, thus, between 6 and 11 samples (mean 9.5) could be scored per accession. In total, 437 individuals from 46 accessions were scored at 186 AFLP loci. Fragment size ranged from 42 to 496 base pairs. The proportion of polymorphic loci and genetic diversity as measured by mean pairwise Jaccard dissimilarities among individuals ranged from 64.5 to 84.9% and from 0.264 to 0.437, respectively. Parameters of genetic diversity for all accessions are listed in Table 1. Genetic diversity parameters did not differ significantly among geographic groups (ANOVA: $P > 0.11$).

In a dendrogram based on pairwise distances among accessions, only Western European accessions formed a larger, well supported cluster (Fig. 2). Within this cluster accessions from Spain aroused as a distinct subgroup among the Irish accessions. Central and Eastern European accessions remained without a clear structuring.

Population differentiation

Genetic differentiation measured as the distances between accessions ranged from 0.37 to 0.62 (mean $D_J = 0.46$). Differentiation among the 46 accessions was significant in all but three comparisons from Central Europe (7/2006 vs. GR 317/1998, 7/2006 vs. GR 367/1997 and 7/2006 vs. 14G0700064). An analysis of molecular variance (AMOVA) revealed that overall differentiation among accession accounted for 24% of the genetic variation ($\Phi_{ST} = 0.24$). The hierarchical AMOVA comprising all four groups of accessions showed that this variation was partitioned to nearly equal amounts among the four groups (11%) and among the accessions within groups (13%, Table 3a). However, results differed when both Western European groups and Central and Eastern European groups of accessions were analyzed separately. Whereas in the first case a large amount of genetic variation resided between groups (18%), this was not the case for the Central and Eastern European groups (1%, Table 3). Also, genetic differentiation as measured by pairwise distances between accessions was significantly higher among all Western European accessions than among Central and Eastern European ones (t test = 7.08, $df = 45.3$, $P < 0.001$). However, a

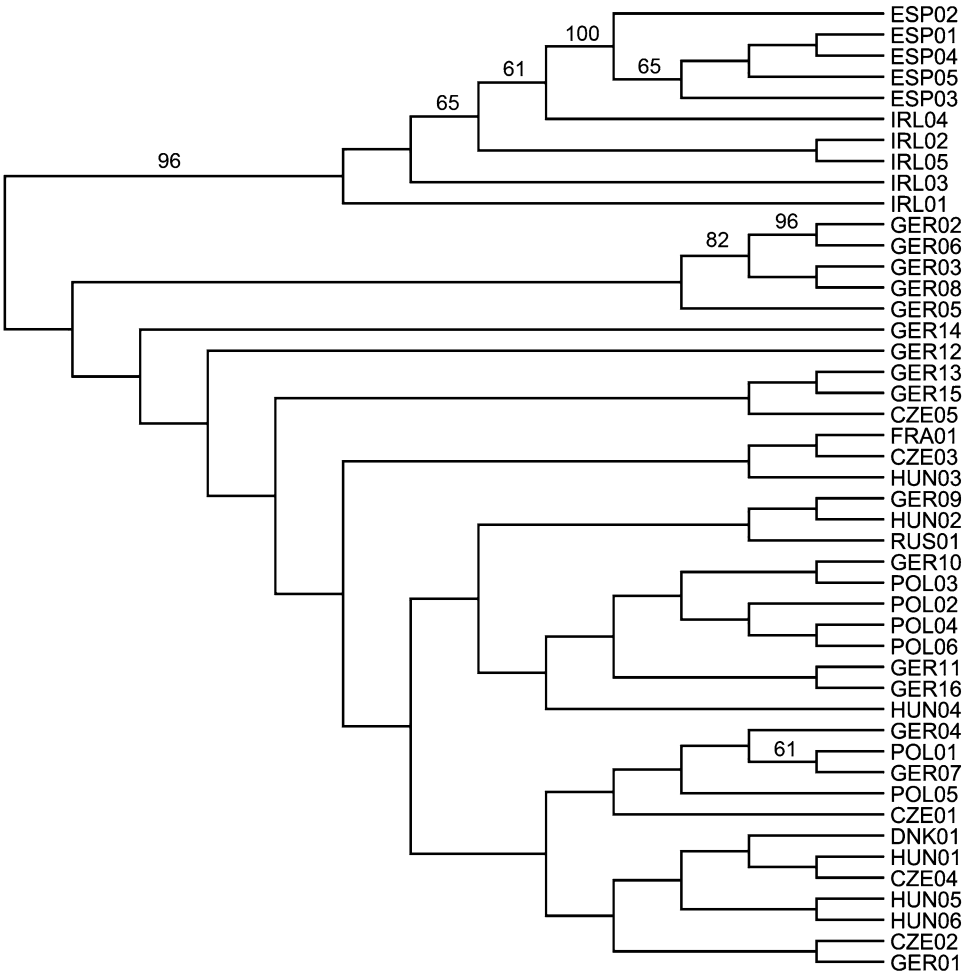

Fig. 2 Dendrogram of 46 accessions of *Arrhenatherum elatius* based on a neighbor-joining analysis of pairwise distances between accessions and computed based on the Jaccard similarity index. *Numbers* above branches indicate bootstrap values (% of 1,000 replicates). Only values larger than 50% are displayed. For population details, see Table 1

similar degree of genetic variation was found within groups of accessions (9 and 15%, respectively).

An isolation-by-distance pattern was found on the global scale when correlating pairwise genetic with pairwise spatial distances between accessions (Spearman's rank correlation $\rho = 0.89$, Mantel $P < 0.001$, Fig. 3). On the regional scale only the Western European accessions showed an Isolation-by-distance pattern ($\rho = 0.67$, Mantel $P < 0.001$), whereas the correlation among Central and Eastern European accessions was not significant (Mantel $P = 0.27$).

Adaptive genetic divergence

By running DFDIST, five out of 186 loci (2.7%) were preselected as loci potentially under selection (Table 4). All five loci showed a much higher degree of genetic differentiation than expected from a neutral drift model ($P > 0.9999$) and thus, are presumably under directional selection. No loci were detected that were under balancing selection, i.e., that showed a lower degree of differentiation than expected. Using PCA, the dimension of environmental parameters was reduced to two factors that summarized 87% of the total variance (Fig. 4). Band frequency of three of the five preselected loci did show a significant negative relationship with the first PCA factor after correction for multiple tests (Table 4). Regression of band frequencies on the second PCA factor revealed two significant relations.

For the more regional scale, DFDIST identified two loci as outliers that were also found on the global scale (ACA/CTA_78 and ACC/CTA_46) as well as two previously undetected loci (ACA/CTA_124 and ACC/CTA_258). However, after Bonferroni correction, the logistic regression of band frequency on spatial and environmental parameters revealed no significant relationships (here, PC 1 and PC 2 accounted for 87% of the environmental variance as well, Table 4).

Discussion

Evaluating the results one has to keep in mind that the accessions investigated were conserved ex situ in gene banks. The genetic composition thus depends on the initial sampling in the natural populations and the number and mode of subsequent regenerations (e.g., Parzies et al. 2000). For example, available information for some accessions

Table 3 Analysis of molecular variance (AMOVA) displaying the genetic variation between individuals, within groups and between (a) the four groups of Spanish, Irish and Central and Eastern European accessions, (b) only between Spanish and Irish accessions and (c) only between Central and Eastern European accessions

Source of variation	*df*	Sum of squares	Percentage of variation
(a)			
Among all four groups	3	9.16	10.80***
Among accessions within groups	42	19.97	12.86***
Within accessions	391	71.68	76.34***
Total	436	100.81	
(b)			
Among the two Western European groups	1	2.78	18.08***
Among accessions within groups	8	3.41	9.20***
Within accessions	92	17.12	72.72***
Total	101	23.30	
(c)			
Among the Central and Eastern European groups	1	0.87	1.04 **
Among accessions within groups	34	16.57	15.07***
Within accessions	299	54.56	83.89***
Total	334	72.00	

*** $P < 0.001$, ** $P < 0.01$

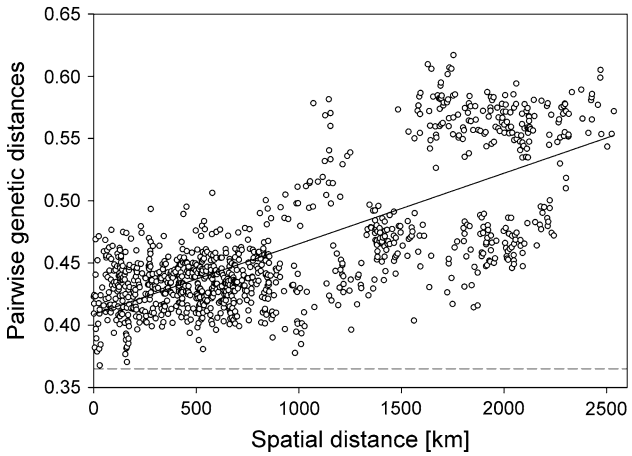

Fig. 3 Scatterplot of pairwise genetic and spatial distances between all sampled accessions of *Arrhenatherum elatius* (Spearman's rank correlation $\rho = 0.89$, Mantel $P < 0.001$). Pairwise genetic distances between accessions were computed as average of all pairwise Jaccard distances between individuals of the respective accessions. The *dashed line* indicates the mean within accession distance between individuals averaged across all accessions ($J = 0.365$)

showed that seed material was initially sampled from 4 to 32 plants (mean 24, $n = 6$). In particular for those accessions with low initial sample size one could expect a bottleneck effect, genetic depauperation and a shift in allelic frequencies by repeated regeneration (Parzies et al. 2000; Soengas et al. 2009). However, the accession with the lowest initial sample size (GR342/1998, $n = 4$) that was regenerated once, did not show a lower level of genetic diversity compared to other accessions. Also, none of the accessions for which data are available underwent more than three regeneration cycles. Furthermore, genetic changes caused by the treatment in gene banks, as for example due to gene flow among simultaneously regenerated accessions, are more likely to obscure present patterns of large-scale differentiation and selective divergence rather than to create such patterns. Thus, it seems valid to use ex situ gene bank accessions of *Arrhenatherum elatius* to represent real populations.

Table 4 Results of logistic regression analyses of band frequency within accessions at potentially adaptive loci on the two main factors representing spatial and environmental parameters

	PC 1		PC 2	
	t value	*P*	*t* value	*P*
All accessions				
ACT/CTT_228	**−4.26**	**>0.001**	−2.36	0.023
ACT/CCT_251	**−8.48**	**>0.001**	−1.03	0.309
ACA/CTA_078	−2.68	0.101	**−3.37**	**0.002**
ACA/CTA_463	**−4.86**	**>0.001**	**−3.42**	**0.001**
ACC/CTA_046	1.66	0.105	−1.61	0.115
Central/Eastern European accessions				
ACA/CTA_078	2.79	0.009	−0.69	0.498
ACA/CTA_124	2.68	0.011	1.85	0.071
ACC/CTA_046	1.89	0.067	0.38	0.706
ACC/CTA_258	−1.37	0.181	1.24	0.222

Analyses were done on the global scale using all accessions and on a more regional scale using Central and Eastern European accessions only. Loci are identified by the selective bases (see Table 2) and fragment size (bp). Significant results after Bonferroni correction are in bold

Genetic diversity within and among accessions

The distribution of genetic diversity within and among populations is largely governed by life-history traits and historical processes affecting the gene exchange between individuals (Barrett and Kohn 1991; Hamrick and Godt 1996; Godt et al. 1998; Pannell and Dorken 2006). Genetic diversity across all individuals of *A. elatius* sampled was similar or even higher than that of other xenogamous grasses. For example, whereas for *A. elatius* the Jaccard dissimilarity between all pairs of individuals ranged from 0.02 to 0.71 (mean $J = 0.46$), values of 0.10–0.64 (mean $J = 0.34$), 0.01–0.31 and 0.13–0.54 have been reported for the outcrossing *Lolium perenne*, *Uniola paniculata* and the dioecious *Poa arachnifera*, respectively (Roldan-Ruiz et al. 2000; Renganayaki et al. 2001; Subudhi et al. 2005).

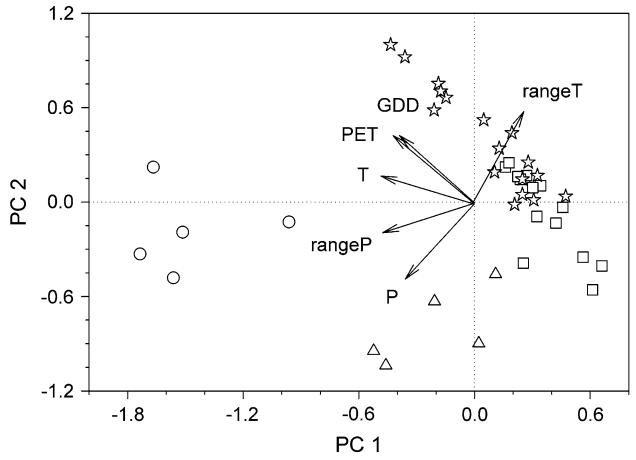

Fig. 4 Representation of a principal component analysis on environmental parameters for all 46 accessions of *Arrhenatherum elatius*. The first two axes account for 87% of the total variance (PC 1: 55%, PC 2: 32%). *Arrows* represent the loadings of the original parameters on the two axes (*P* precipitation, *T* temperature, *GDD* growing degree days, *PET* potential evapotranspiration, *rangeT* range of the mean monthly temperatures, *rangeP* range of the monthly precipitation). For *symbols* see Fig. 1

Among accessions and regions sampled, *A. elatius* exhibited a similar level of genetic diversity suggesting homogeneity in population characteristics. Assuming reduced sexual reproduction in colder climates (Pfitzenmeyer 1962), one could expect a decline in genetic diversity with decreasing mean annual temperatures or GDD. Indeed, an additional analysis showed that genetic diversity (*J*) of *A. elatius* accessions correlated with the number of GDD ($r = 0.33$, $P = 0.026$). However, as the degree of inbreeding in *A. elatius* has not been determined across the sampled regions, it remains unclear whether this is due to changes in the mating system or due to other reasons like bottlenecks.

The effects of spatial isolation between subpopulations are likely to be less pronounced in species that exhibit high levels of gene flow by seeds and pollen (Charlesworth 2003). Thus, outcrossing species are expected to show a lower differentiation among populations than selfing species. Compared to most other polyploid grasses investigated by AFLP markers, *A. elatius* showed a higher differentiation among accessions, but similar levels as, for example, the outcrossing *Agrostis capillaris* (Table 5). Even neighboring accessions were found to be significantly differentiated. These results are supported by the AMOVA that found a significant part of the genetic variation among accessions (24%). Using allozyme data, for *A. elatius* the inbreeding coefficient *F* has been shown to vary between −0.1 and 0.9 with higher values in more dense populations (Ducousso et al. 1990). The authors argue that a reduction in the size of the genetic neighborhood leads to increased inbreeding in more dense populations but not selfing. Thus, in spite of a considerable pollen flow among individuals, this phenomenon may lead to increased genetic differentiation. Similar to the effects of selfing, also the occurrence of double reduction can promote differentiation among populations due to a reduction in effective population size (Ronfort et al. 1998).

An overall increase of genetic differentiation between accessions with increasing distance was found (Fig. 3), which would be expected if the populations were at gene flow–drift equilibrium. However, at regional equilibrium, the degree of scatter should also increase with spatial distances (Hutchison and Templeton 1999), which was not the case. Furthermore, the pattern was not consistent across regions. Genetic and geographical distances were uncorrelated among Central and Eastern European accessions. Given the strong differentiation among accessions this suggests only a limited influence of gene flow within this region. Thus, the significant pattern found at the large scale is likely to primarily reflect species history rather than recent gene flow–drift equilibrium. This is substantiated by the similarity analysis. Despite the strong differentiation among and the high genetic diversity within accessions a statistically significant clustering occurred only between Western European accessions on the one hand and Central/Eastern European accessions on the other (Fig. 2). Within the large number of Central and Eastern European accessions, no further groupings could be distinguished.

This lack of regional structure across large parts of Central and Eastern Europe may be related to human activity. With the agricultural reforms at the end of the eighteenth century

Table 5 Genetic differentiation among accessions of some polyploid grasses investigated using AFLP markers

Species	Reproduction	Ploidy	D_J (Range)	D_J (Mean)	Range of study	Reference
Bromus catharticus	Selfing	6*x*	<0.02		South America, cultivars	Puecher et al. (2001)
Cynodon dactylon	Clonal/selfing	4*x*/6*x*	0.02–0.47	0.30	Asia, Europe, Africa, Australia	Wu et al. (2004)
Uniola paniculata	Outcrossing	4*x*	0.02–0.24		Southeastern United States	Subudhi et al. (2005)
Trichloris crinita	Selfing/apomictic	4*x*	0.08–0.69		South America	Cavagnaro et al. (2006)
Aegilops ssp. ($n = 5$)	Selfing	4*x*/6*x*	0.10–0.37		Spain	Monte et al. (2001)
Agrostis capillaris	Outcrossing	4*x*	0.30–0.66	0.49	Northern Hemisphere, cultivars	Zhao et al. (2006)
A. elatius	Mixed mating	4*x*	0.37–0.62	0.46	Europe	This study

Genetic differentiation is described by the range and mean of pairwise Jaccard dissimilarities between accessions (D_J)

and in the beginning and the nineteenth century the seed import of grassland species and the trading of seed material within Central and Eastern Europe strongly increased (Kauter 2002). A natural spread and colonization of the species from one source of origin may rather have led to a clustering of accessions based on their geographical origin. However, phylogeographical studies using maternally inherited markers and the inclusion of a larger sample of potential French source regions are necessary to disclose possible relationships and ways of introduction (e.g., Blum et al. 2007). Given the potential anthropogenic influence on grassland species in general, it is unlikely that the patterns of genetic structuring described here for *A. elatius* can be generalized. This is substantiated by a study on the grassland species *Festuca pratensis* and *Lolium multiflorum* that showed marked differences in their genetic structuring both in neutral and adaptive traits on the regional scale (Peter-Schmid et al. 2008).

Divergent selection

Considerable morphological and phenological variability has been described in *A. elatius* (Sulinowski 1965a; Petit et al. 1997; Petit and Thompson 1998) and some polymorphisms might be genetically controlled (Mahmoud et al. 1975; Cuguen et al. 1989). In a common garden experiment comprising *A. elatius* populations from southern France, Petit and Thompson (1998) demonstrated a significant genotype effect in stem height, date of initial flowering and leaf surface area in some of their populations studied. The authors suggested that variation in a number of traits is adaptive and reflects different selection regimes in different environments. Ducousso et al. (1990) found a higher neutral genetic and morphological diversity in populations of *A. elatius* from heavy metal contaminated soils compared to adjacent populations from non-toxic soil. It was argued that this pattern is possibly a result of diversifying selection on toxic soils due to an increased heterogeneity in these habitats. Also, between neighboring heavy metal tolerant and nontolerant populations of *A. elatius* prezygotic mating barriers have been reported (Lefebre and Vernet 1990). Thus, despite a potentially strong gene flow by means of wind pollination, *A. elatius* seems to be able to respond to different selection regimes on smaller scales.

In this study, four AFLP loci were found on the global scale that exhibited a higher genetic differentiation than expected under a neutral model. Furthermore, band frequencies were correlated significantly with spatial and environmental parameters (Table 4). However, it has been suggested that genome scan methodologies as used here may underestimate the number of selected loci (see Teshima et al. 2006). Additionally, autotetraploidy exacerbates the identification of loci under selection or are linked to selected genes. Thus, the percentage of loci detected in *A. elatius* should not be compared to other studies (e.g., Mealor and Hild 2006; Nosil et al. 2009). Nevertheless, the correlation found between band frequency and spatial and environmental parameters at the four loci showed that, on the global scale, genetic differentiation in *A. elatius* is also the result of adaptive processes. Although only few environmental variables were included in our analysis, the results show that parameters related to temperature and precipitation are very likely to exert selective pressures in *A. elatius*. Here, the four loci identified can provide a starting point for the search for genes involved in adaptation (e.g., Wood et al. 2008). Furthermore, information on the relative distribution of certain alleles in individuals and proveniences could be valuable for interpreting data from climate change experiments that, for example, are focusing on the effects of altered precipitation patterns on grassland related ecosystem services (Jentsch et al. 2007, 2009; Kreyling et al. 2008). Such experiments will show if proveniences selected for the presence of potentially adaptive loci indeed enhance the adaptation to the expected future climate conditions. In this case, an effective adaptation against adverse effects of climate change on ecosystems could apply assisted migration of ecotypes rather than species. This would have the great advantage of building climate-safe communities without changing species identity, at least for widely distributed key species.

In conclusion, this study demonstrated high genetic variability in *A. elatius*. On the European scale, groups of Western European and Central/Eastern European accessions were clearly separated by the AFLP markers used. No further regional patterns of genetic structure could be detected. However, at the same time we detected strong genetic differentiation among local populations, which is concordant with the phenotypic variability and differentiation observed in this species and suggests genetic isolation among populations. Thus, the differentiation among populations and the fact that the differentiation detected is partly adaptive may indicate local rather than regional adaptation in *A. elatius* despite a potentially rather recent history of introduction. Consequently, adaptation should be investigated and seed sources should be developed at more local scales.

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